

Molt-related Susceptibility and
Regenerative Limb Growth as Sensitive
Indicators of Aquatic Pollutant
Toxicity to Crustaceans

University of West Florida, Pensacola

Prepared for

Environmental Research Lab., Gulf Breeze, FL

1986

TECHNICAL REPORT DATA

(Please read instructions on the reverse before completing)

1. REPORT NO. EPA/600/D-86/108		2.	3. PB86-213741
4. TITLE AND SUBTITLE MOLT-RELATED SUSCEPTIBILITY & REGENERATIVE LIMB GROWTH AS SENSITIVE INDICATORS OF AQUATIC POLLUTANT TOXICITY TO CRUSTACEANS		5. REPORT DATE 1986	
7. AUTHOR(S) K.R. Rao and P.J. Conklin		6. PERFORMING ORGANIZATION CODE	
9. PERFORMING ORGANIZATION NAME AND ADDRESS University of West Florida Department of Biology Pensacola, FL 32504		8. PERFORMING ORGANIZATION REPORT NO.	
12. SPONSORING AGENCY NAME AND ADDRESS Environmental Research Laboratory - Gulf Breeze, FL Office of Research and Development U.S. Environmental Protection Agency Gulf Breeze, FL 32561		10. PROGRAM ELEMENT NO.	
		11. CONTRACT/GRANT NO. 807417 & 804541	
		13. TYPE OF REPORT AND PERIOD COVERED	
		14. SPONSORING AGENCY CODE EPA/600/4	
15. SUPPLEMENTARY NOTES In: "Indian Ocean -- Biology of Benthic Marine Organisms and Techniques & Methods as Applied to the Indian Ocean. 1986. Oxford & IBH Publishing Co.			
16. ABSTRACT M. Thompson, R. Sarojini, & R. Nagabhushanam, Eds., p. 523-534. This study evaluated the comparative toxicity of various pollutants to intermolt and molting grass shrimp (<i>Palaemonetes pugio</i>). Most of the tested materials (pentachlorophenol, tetrachlorophenols, trichlorophenols, methylenebis dichlorophenol, dibutyl phthalate, chromium, and drilling mud) were more toxic to molting shrimp than to intermolt shrimp. Radio-tracer studies with 2,4,5-trichlorophenol and pentachlorophenol indicated that the increased susceptibility of newly molted shrimp is linked to increased pollutant uptake. Additional work showed that certain chlorophenols, dithiocarbamates, dibutyl phthalate, and chromium cause inhibition of regenerative limb growth in grass shrimp without affecting the molt cycle duration. The median effective concentrations (EC ₅₀ s) for intermolt shrimp. Thus, limb regeneration assays with intermolt shrimp as well as toxicity tests with molting shrimp serve as sensitive indicators of aquatic pollutant toxicity.			
17. KEY WORDS AND DOCUMENT ANALYSIS			
a. DESCRIPTORS	b. IDENTIFIERS: OPEN ENDED TERMS	c. COSATI Field/Group	
REPRODUCED BY NATIONAL TECHNICAL INFORMATION SERVICE U.S. DEPARTMENT OF COMMERCE SPRINGFIELD, VA. 22161			
18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC		19. SECURITY CLASS (This Report) UNCLASSIFIED	21. NO. OF PAGES 13
		20. SECURITY CLASS (This page) UNCLASSIFIED	22. PRICE 5

Reprint from

———— INDIAN OCEAN ————

Biology of Benthic Marine Organisms

Techniques and Methods as Applied to the Indian
Ocean

Editors

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OXFORD & IBH PUBLISHING CO.
New Delhi Bombay Calcutta

54. Molt-related Susceptibility and Regenerative Limb Growth as Sensitive Indicators of Aquatic Pollutant Toxicity to Crustaceans

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This study evaluated the comparative toxicity of various pollutants to intermolt and molting grass shrimp (*Palaemonetes pugio*). Most of the tested materials (pentachlorophenol, tetrachlorophenols, trichlorophenols, methylenebis dichlorophenol, dibutyl phthalate, chromium, and drilling mud) were more toxic to molting shrimp than to intermolt shrimp. Radiotracer studies with 2,4,5-trichlorophenol and pentachlorophenol indicated that the increased susceptibility of newly molted shrimp is linked to increased pollutant uptake. Additional work showed that certain chlorophenols, dithiocarbamates, dibutyl phthalate, and chromium cause inhibition of regenerative limb growth in grass shrimp without affecting the molt cycle duration. The median effective concentrations (EC_{50} s) for inhibition of limb regeneration were well below the median lethal concentration (LC_{50} s) for intermolt shrimp. Thus, limb regeneration assays with intermolt shrimp as well as toxicity tests with molting shrimp serve as sensitive indicators of aquatic pollutant toxicity.

INTRODUCTION

Recent work in our laboratory has focused on physiological, biochemical, and histopathological evaluations of the toxicity of pollutants such as pentachlorophenol (Rao 1978, Rao et al. 1979), dichlorophenols, trichlorophenols, tetrachlorophenols (Rao et al. 1981), dithiocarbamates (Conklin and Rao 1982; Rao et al. 1982; Doughtie and Rao 1983a, b), drilling muds (Conklin et al. 1980, 1983), and chromium (Doughtie and Rao, unpublished; Doughtie et al. 1983, Rao et al., unpublished). During the course of these and other studies dealing mainly with the grass shrimp (*Palaemonetes pugio*), we paid particular attention to molting and related phenomena. In view of the known molt-related cyclic changes in cuticular/epithelial permeability in Crustacea (Passano 1960, Mantel and Farmer 1983), we evaluated the relative toxicity of diverse pollutants to intermolt and molting shrimp and also examined the uptake of certain pollutants by shrimp in different molt stages. The results of these studies, as well as the evaluations of regenerative limb growth as a sublethal bioassay for aquatic pollutants, are summarized in this report.

MATERIAL AND METHODS

Chemicals

Analytical grade (99 percent pure) 2,4-dichlorophenol; 94 percent pure 2,4,5-trichlorophenol; 98 percent pure 2,4,6-trichlorophenol; 98 percent pure 2,3,4,5-tetrachlorophenol; 98 percent pure 2,3,5,6-tetrachlorophenol; and 99 percent pure pentachlorophenol were obtained from the Aldrich Chemical Company. Technical grade 2,3,4,6-tetrachlorophenol was obtained from Fluka AG. Each of these chlorophenols was initially dissolved in ethanol, and suitable amounts of this stock solution were added to filtered seawater to obtain the desired test concentrations.

A complex chlorophenol formulation, Surflo B-33, composed of 25 percent 2,2'-methylenebis (4,6)-dichlorophenol and sodium salts of other chlorophenols (from Baroid Corporation) was also tested. This material was dissolved in seawater and then serially diluted.

The dithiocarbamates employed were Aquatreat DNM-30 (active ingredients: 15 percent sodium dimethyldithiocarbamates plus 15 percent disodium ethylene bisdithiocarbamate) from Alco Chemical Corporation (Chattanooga Tennessee), and Busan-85 (active ingredient: 50 percent potassium dimethyl-dithiocarbamate) from Buckman Laboratories (Memphis, Tennessee). Since both products, as well as Busan-30 (a benzothiazole formulation from Buckman Laboratories), were aqueous solutions, test stock solutions were prepared by volumetric dilution.

The compound dibutyl phthalate, obtained from Aldrich Chemical Company, was initially dissolved in acetone and then suitable aliquots were diluted volumetrically with seawater. Test solutions of hexavalent chromium (Cr^{6+}) were prepared by dissolving appropriate amounts of sodium chromate in seawater. In tests with oil, the desired amount of No. 2 fuel oil (American Petroleum Institute's reference sample) was mixed with seawater and tested as an oil-water dispersion.

A sample of drilling mud (fluid) was obtained from the Mobile Oil Company well No. 1-76 located in Mobile Bay, Alabama. This sample of water-based used mud, designated as Mud IX (for details, see Conklin et al. 1983), had a density of 1.16 g/ml and had considerable amounts of No. 2 fuel-oil-like petroleum hydrocarbons (4.77 mg/g whole mud). Test solutions were prepared by diluting the appropriate volume of whole drilling mud with seawater.

In all cases, the seawater employed for preparing solutions was filtered (5 μm pore size) and then diluted to a salinity of 10 ppt with distilled water. The stock solutions and the appropriate dilutions were prepared fresh, as needed.

Grass shrimp

Adult grass shrimp (*P. pugio*) collected from grass beds located in Santa Rosa Sound, Gulf Breeze, Florida, were maintained in the laboratory in glass aquaria containing aerated, recirculating seawater and an oyster-shell biological filter. The shrimp were fed a daily diet of freshly hatched brine shrimp (*Artemia*) supplemented with Tetramin staple fish food flakes. All tests were conducted with non gravid shrimp in known stages of the molt cycle. The molt stage of each shrimp was determined by microscopic examination of the outer uropod edges for evidence of apolysis (epidermal retraction) and formation of new setae (Conklin and Rao 1978).

General experimental conditions

Grass shrimp were exposed individually in glass jars containing 250 ml of test medium or seawater, alone, in the case of controls. The solutions were not aerated but were renewed every 24 hr to provide fresh oxygenated media as well as to remove accumulated waste products. The dissolved oxygen content and pH of the solutions were determined initially and just prior to changing solutions. The salinity (10 ppt), temperature ($20 \pm 1^\circ\text{C}$), and pH (7.8 to 8.0) were the same for all experiments. A controlled incubator was used to maintain the required temperature while a 12-hr light/12-hr dark photoperiod was provided by cool-white fluorescent illumination. The shrimp were not fed during the 96-hr toxicity tests but were fed live brine shrimp nauplii on alternate days during prolonged exposures.

Acute toxicity tests

Intermolt (Stage C) and molting shrimp were used in separate tests. The latter shrimp were in late premolt (Stages D₂ to D₄) at the start of the test and had undergone molting (ecdysis) during the 96-hr test period. In tests with a given compound, a minimum of 20 shrimp of the desired molt stage were exposed to each test concentration and also as seawater controls. At 24-hr intervals, prior to changing solutions, the test jars were examined for cast exuvia and for shrimp mortalities.

Limb regeneration

Since adequate time prior to ecdysis is required for successful limb bud growth (Rao et al. 1978), regeneration tests were initiated using only intermolt shrimp (Stage C). At each test concentration, 20 to 24 shrimp were used. Following removal of the left fifth pereopod, the shrimp were placed in either experimental or control media. On the fourth, seventh and ninth day after limb removal, the shrimp were examined microscopically to assess limb bud growth and progress in the molt cycle. To compare regenerative growth for different shrimp, a regenerative index (R-value) was calculated according to the formula of Bliss (1956):

$$R\text{-value} = \frac{\text{length of limb bud}}{\text{length of carapace}} \times 100$$

The R-values from any shrimp that molted prior to the twelfth day were not included in the computation of the average R-values.

Radiotracer studies

Uniformly labeled ^{14}C -2,4,5-trichlorophenol (specific activity: 12.12 mCi/mM) and ^{14}C -pentachlorophenol (specific activity: 10.04 mCi/mM, 10.62 mCi/mM), obtained from Pathfinder Laboratories, St. Louis, were used. In separate experiments, intermolt (Stage C) shrimp and newly molted shrimp (Stage A, within five minutes after molting) were exposed to filtered seawater containing 1.0 ppm 2,4,5-trichlorophenol (0.118 ppm labeled + 0.882 ppm unlabeled) or 2.0 ppm pentachlorophenol (0.266 ppm labeled + 1.734 ppm unlabeled) for a one-hour period. Subsequently, the shrimp were transferred to a seawater wash for two minutes, then blotted dry and weighed individually. Some of the shrimp were processed as a whole. Others were dissected and each of the several tissues (abdomen, hepatopancreas, digestive tract, cephalothorax) was weighed and processed separately. The shrimp or their tissues were placed in scintillation vials containing up to 1.0 ml of Protosol tissue solubilizer (New England Nuclear), then macerated and digested overnight in a shaking water bath at 50°C. Following digestion, samples were neutralized with acetic acid; then 15 ml of Aquasol (New England Nuclear) was added to each sample. The radioactivity of these samples was determined by using a Beckman LS-133 liquid scintillation counter. Background and quench corrections were made using control (unexposed) animals. Quench correction for counting efficiency was made by internal standard method.

Statistical analysis

Data analysis was performed using the statistical analysis (SAS) computer package (Barr et al. 1976). The 96-hr LC_{50} s and their corresponding 95 percent fiducial limits were computed from the acute toxicity data using the SAS probit procedure, whereas the limb regeneration data were analyzed using the SAS General Linear Model (GLM) procedure and Duncan's Multiple Range Test to evaluate significant ($P=0.05$ level) effects within treatments. The EC_{50} s for inhibition of limb bud growth were computed using the probit procedure.

RESULTS AND DISCUSSION

Comparative toxicity

Among the tested materials, dithiocarbamates were the most toxic and

the drilling mud sample least toxic to grass shrimp (table 1). From a comparative taxonomic point of view, however, the LC₅₀s derived from tests with grass shrimp, coupled with previous work on other species, indicate that pollutants such as drilling muds (Neff et al. 1981; Conklin et al. 1980, 1983), oil (Anderson et al. 1974), chromium (Rao et al., unpublished) as well as dithiocarbamates (Conklin and Rao 1982, Rao et al. 1982) are generally more toxic to crustaceans than to other invertebrates or fish. Chlorophenols were once thought to be more toxic to fish than to crustaceans but recent work indicates that they are equitoxic to representative species from both groups (Rao 1978, Rao et al. 1981).

The relative toxicities of compounds, within a given chemical group seem to vary with the species tested. In the case of chlorophenols, for example, toxicity was reported to increase in relation to the degree of chlorination when tested on rats (Farquharson et al. 1958, Ahlborg and Larsson 1978) and fish (Ingolls et al. 1966, Kobayashi 1979). Tests on the crayfish *Astacus fluviatilis* showed, however, that 2,3,6-trichlorophenol is more toxic than pentachlorophenol (Kaila and Saarikoski 1977). Similarly, our tests with intermolt grass shrimp showed that certain lesser chlorinated phenols (2,4,5-trichlorophenol and 2,3,4,5-tetrachlorophenol) are more toxic than pentachlorophenol (table 1).

Whereas 2,4-dichlorophenol and dithiocarbamates were each equitoxic to intermolt and molting shrimp, the other tested materials were more toxic to molting shrimp than to intermolt shrimp. There was a two- to sixfold difference between the LC₅₀s for the two kinds of shrimp (table 1). Studies on the toxicity of chromium to *Daphnia pulex* (Lee and Buikema 1979), of chlorine to *Penaeus kerathurus* (Saroglia and Scarno 1979), and of copper and zinc to *Crangon crangon* (Price and Uglow 1979) also revealed increased susceptibility of crustaceans to aquatic pollutants at the time of (or shortly after) molting.

To assess whether this molt-related susceptibility is linked to variations in pollutant uptake, we studied the accumulation of ¹⁴C-2,4,5-trichlorophenol (figure 1) and ¹⁴C-pentachlorophenol (figure 2) in intermolt and newly molted grass shrimp. The latter shrimp accumulated these chlorophenols to a greater extent than did the intermolt shrimp. This difference in accumulation was evident not only at whole-body residue level but also at tissue levels of these chlorophenols (figures 1, 2).

The magnitude of relative increase in pentachlorophenol accumulation in newly molted shrimp (figure 2) was much greater than that noted with 2,4,5-trichlorophenol accumulation (figure 1). Interestingly, among the chlorophenols exhibiting an increase in toxicity to grass shrimp at the time of molting, pentachlorophenol showed the greatest increase (5.68-fold) in molt-related toxicity, whereas 2,4,5-trichlorophenol showed the least increase (1.75-fold). This correlation between relative increase in

Table I. Comparative toxicity and regeneration-inhibiting potency of diverse pollutants in tests with grass shrimp (*Palaemonetes pugio*).

Chemical	Toxicity		Inhibition of limb regeneration: EC ₅₀ (95% fiducial limits) ppm
	96-hr LC ₅₀ (95% fiducial limits), ppm	Molting Shrimp	
Chlorophenols			
Pentachlorophenol	2.50 (1.91-3.29)	0.44 (0.18-0.67)	0.57 (0.42-0.75)
2,3,4,5-Tetrachlorophenol	0.86 (0.73-0.98)	0.37 (0.35-0.39)	0.30 (0.15-0.54)
2,3,4,6-Tetrachlorophenol	3.70 (2.98-5.25)	0.81 (0.61-0.89)	0.78 (0.63-0.94)
2,3,5,6-Tetrachlorophenol	4.10 (3.30-5.31)	1.17 (1.08-1.27)	Not tested
2,4,5-Trichlorophenol	1.12 (0.92-1.43)	0.64 (0.36-0.80)	No dose-related effect
2,4,6-Trichlorophenol	3.95 (3.28-4.95)	1.21 (1.11-1.31)	No dose-related effect
2,4-Dichlorophenol	2.55 (2.28-2.86)	2.16 (1.49-2.73)	Not tested
Surflo B-33*	14.28 (12.42-16.35)	2.14 (2.03-2.24)	0.89 (0.73-1.01)
Dithiocarbamates			
Aquaatec DNM-30*	0.112 (0.103-0.172)	0.127 (0.113-0.145)	0.061 (0.046-0.072)
Busan-85*	0.056 (0.042-0.070)	0.019 (0.042-0.051)	0.005 (0.004-0.006)
Benzothiazole			
Busan-30*	0.260 (0.227-0.290)	0.144 (0.131-0.160)	Not tested
Other Compounds			
Dibutyl phthalate	16.46 (14.34-20.92)	8.58 (7.39-10.62)	7.01 (6.42-7.61)
Chromium (Cr ⁶⁺)	14.63 (12.93-16.32)	4.86 (3.92-5.78)	0.38 (0.12-0.64)
API No. 2 Oil	5.86 (5.05-6.96)	4.24 (3.73-4.65)	Not tested
Drilling Mud*	2.409 (1.761-3.198)	5.40 (470-620)	Not tested

*For composition see Material and Methods.

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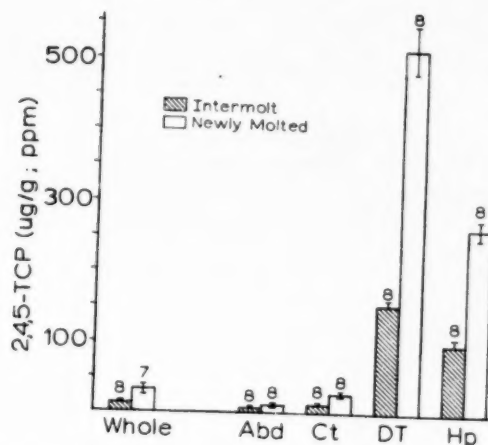


Figure 1. Accumulation of 2,4,5-trichlorophenol (TCP) in the whole body and in the abdomen (Abd), cephalothorax (Ct), digestive tract (DT: includes the stomach, midgut, and hindgut), and hepatopancreas (Hp) of intermolt (Stage C) and newly molted (within five minutes after molting) grass shrimp, *Palaemonetes pugio*. The shrimp were exposed to 1.0 ppm 2,4,5-trichlorophenol (0.118 ppm ^{14}C -2,4,5-TCP plus 0.882 ppm unlabelled 2,4,5-TCP) for a period of one hour. The amounts of TCP accumulated in the whole body and in the various tissues were estimated on the basis of radioactivity measurements. Each bar represents the mean $\pm$ standard errors. The number of animals or of tissue samples used is shown on top of each bar. (From Rao et al. 1981.)

toxicity and relative increase in bioaccumulation of chlorophenols suggests that the molt-related increase in susceptibility is linked to increased pollutant uptake.

Regenerative limb growth

Change in growth rate represents an important measure of organismic performance as it represents an ecologically-relevant integrated response (Sastry and Miller 1981). Crustacean limb regeneration, being a molt-related phenomenon (Bliss 1956) and having multiple phases (wound healing, cell division, cell differentiation, tissue growth) (Adiyodi 1972), can serve as a useful external indicator of pollutant-induced stress.

In our studies with glass shrimp, a majority of the tested compounds (pentachlorophenol, tetrachlorophenols, Surflo B-33, dithiocarbamates, dibutyl phthalate, chromium) caused dose-related inhibition of regenerative limb growth. As an example, the dose-related inhibition elicited by chlorophenols is shown in figure 3. The median effective concentrations (EC_{50}s) for this inhibition, presented in table 1, were computed from the regeneration indexes (R-values) on the ninth day after limb removal.

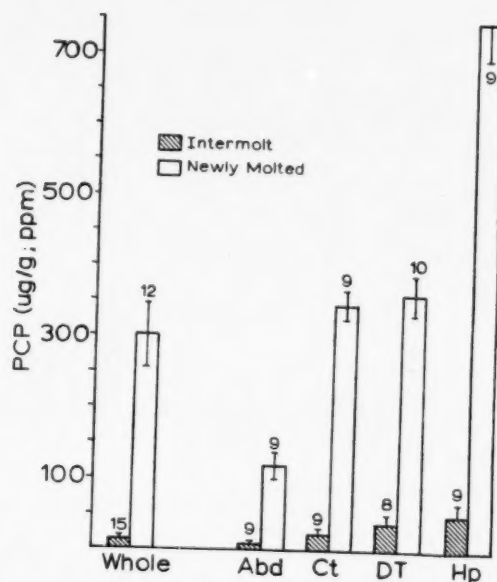


Figure 2. Accumulation of pentachlorophenol (PCP) in the whole body and in the abdomen (Abd), cephalothorax (Ct), digestive tract (DT), and hepatopancreas (Hp) of intermolt (Stage C) and newly molted grass shrimp, *Palaemonetes pugio*. The shrimp were exposed to 2.0 ppm PCP (0.226 ppm ^{14}C -PCP plus 1.734 ppm unlabelled PCP) for one hour. The amounts of PCP accumulated in the whole body and in the various tissues were estimated on the basis of radioactivity measurements. Each bar represents the mean $\pm$ standard errors. The number of shrimp or of tissue samples used is shown on top of each bar. (From Rao et al. 1981.)

The EC_{50} s for inhibition of regeneration were in all cases lower (two to four times) than the median lethal concentrations (LC_{50} s) for intermolt shrimp. In the case of pentachlorophenol, tetrachlorophenols, and dibutyl phthalate, the EC_{50} s for inhibition of regeneration were nearly identical to the LC_{50} s for molting shrimp. In the case of Surflo B-33, dithiocarbamates, and chromium (Cr^{6+}), the EC_{50} s for regeneration inhibition were lower (2 to 13 times) than the LC_{50} s for molting shrimp. This shows that the limb regeneration assays are generally more sensitive than acute toxicity tests with intermolt shrimp; the former seem to be as sensitive or more sensitive than toxicity tests with molting shrimp. Thus, regenerative limb growth serves as a sensitive sublethal bioassay for evaluating the stress induced by aquatic pollutants.

In the studies with grass shrimp, the inhibition of limb regeneration was not accompanied by any alteration in the length of the intermolt cycle. This suggests that inhibitory effects are exerted directly on the developing

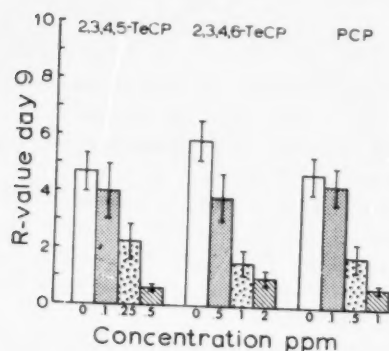


Figure 3. Effects of tetrachlorophenols (TeCP) and pentachlorophenol (PCP) on regeneration of the left fifth pereiopod in the grass shrimp, *Palaemonetes pugio*. The R values (mean $\pm$ standard errors) were derived from the measurements of limb bud size on the ninth day after limb removal. The control shrimp (open bars) were maintained in seawater; the experimental shrimp were exposed to chlorophenol-containing seawater beginning the day of limb removal until the completion of ecdysis. The shrimp were fed during these experiments. (Excerpts from Rao et al. 1981.)

limb bud tissues, rather than being mediated through factors controlling the molt cycle.

However, in tests with fiddler crabs, DDT accelerated the rate of limb regeneration and shortened the time to molting (Weis and Mantel 1976), whereas heavy metals (Weis 1976, 1977; Weis and Weis 1979) and polychlorinated biphenyls (Fingerman and Fingerman 1979, 1980) inhibited regenerative limb growth and lengthened the time to molting. Similarly, in the blue crabs, benzene retarded regenerative limb growth and delayed molting (Cantelmo et al. 1982). Thus, in fiddler crabs and blue crabs, the observed effects may be a result of pollutant-induced alteration in the function of the neuroendocrine system and modification of the molting cycle.

In view of the diverse mechanisms of pollutant effects on regeneration, and its demonstrated relative sensitivity, crustacean limb regeneration merits recognition as an important sublethal assay for aquatic pollutants. This assay, as well as molt-related susceptibility, contributes to an increased sensitivity of toxicity tests involving crustaceans.

Studies reported were supported by grants R-804541 and CR-807417 from the U.S. Environmental Protection Agency. We are thankful to Thomas W. Duke, U.S. Environmental Research Laboratory, Gulf Breeze, for his support and encouragement during this investigation.

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